Reduced Hippocampal Brain-Derived Neurotrophic Factor (BDNF) in Neonatal Rats after Prenatal Exposure to Propylthiouracil (PTU)

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Thyroid hormone is critical for central nervous system development. Fetal hypothyroidism leads to reduced cognitive performance in offspring as well as other effects on neural development in both humans and experimental animals. The nature of these impairments suggests that thyroid hormone may exert its effects via dysregulation of the neurotrophin brain-derived neurotrophic factor (BDNF), which is critical to normal development of the central nervous system and has been implicated in neurodevelopmental disorders. The only evidence of BDNF dysregulation in early development, however, comes from experimental models in which severe prenatal hypothyroidism occurred. By contrast, milder prenatal hypothyroidism has been shown to alter BDNF levels and BDNF-dependent functions only much later in life. We hypothesized that mild experimental prenatal hypothyroidism might lead to dysregulation of BDNF in the early postnatal period. BDNF levels were measured by ELISA at 3 or 7 d after birth in different regions of the brains of rats exposed to propylthiouracil (PTU) in the drinking water. The dose of PTU that was used induced mild maternal thyroid hormone insufficiency. Pups, but not the parents, exhibited alterations in tissue BDNF levels. Hippocampal BDNF levels were reduced at both d 3 and 7, but no significant reductions were observed in either the cerebellum or brain stem. Unexpectedly, more males than females were born to PTU-treated dams, suggesting an effect of PTU on sex determination. These results support the hypothesis that reduced hippocampal BDNF levels during early development may contribute to the adverse neurodevelopmental effects of mild thyroid hormone insufficiency during pregnancy. (*Endocrinology* 153: 1311–1316, 2012)

Thyroid hormones (T_3 and T_4) are crucial for normal central nervous system (CNS) development. Before the onset of fetal thyroid function in humans and rats, the maternal thyroid gland is the only source of T_4 for the fetus, in which it is deiodinated to the biologically more potent T_3 (1).

Maternal T_4 insufficiency has serious adverse consequences for the neurodevelopment of the offspring, including irreversible cognitive deficits (2–4). Even modest reductions in maternal T_4 levels can result in impaired neurological development in children (5). Moreover, impaired neurodevelopment may occur after reduced maternal T_4, even if circulating maternal T_3 and TSH levels are normal (3). Infants may exhibit impaired cognitive development and other debilitating conditions such as cerebral palsy, even when maternal T_4 is decreased only temporarily, as in transient hypothyroxinemia of prematurity (6, 7).

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Abbreviations: BDNF, Brain-derived neurotrophic factor; CNS, central nervous system; P, postnatal day; PTU, propylthiouracil.

In rats, feeding iodine-deficient diets to create hypothyroidism results in neurodevelopmental defects (8–10). Cortical (9), hippocampal (10), and cerebellar (8) development are all abnormal in pups born to iodine-deficient dams. These studies, and observations in humans, have led to the suggestion that neurodevelopmental defects in children living in areas with severe iodine deficiency could be caused by fetal thyroid hormone insufficiency (11–14).

The mechanisms underlying the effects of hypothyroidism on the developing brain are not completely understood. Growing evidence suggests a role for brain-derived neurotrophic factor (BDNF), a neurotrophin that is critical to normal development of the CNS and also regulates synaptic transmission, dendritic structure, and synaptic plasticity in adulthood (15). In rats, maternal thyroidectomy significantly reduces BDNF expression in the brains of developing rat pups (16). The effects of milder hypothyroidism, however, are uncertain. Understanding the effects of subclinical hypothyroidism is important, not only because of the neurodevelopmental concerns (3, 16) but also because many environmental chemicals interfere with thyroid hormone action (17–19). Rising rates of neurodevelopmental disorders such as autism may reflect the prevalence of these chemicals, making the issue a potential public health concern (19).

Mild thyroid hormone insufficiency in rats does not alter BDNF levels in the hippocampus at postnatal d 14 (20). The present study was performed to test the hypothesis that there might be BDNF deficits earlier, during the first postnatal week, which is important because this time period is critical for the developing CNS. To impair thyroid function in a manner comparable with earlier studies (20), we administered propylthiouracil (PTU) to dams. PTU inhibits both the synthesis of thyroid hormones in the thyroid gland and the conversion of T_4 to its active form, T_3 (21). In development, PTU has been shown to reduce growth in the offspring and induce long-term structural and functional brain deficits (22–28), even at doses that produce only a relatively mild, transient hypothyroidism in the mothers (22, 29).

Our results indicate that such treatments alter BDNF levels in the early postnatal brain in a region-specific manner: hippocampal BDNF is reduced, whereas BDNF concentrations in the cerebellum and brain stem remain unchanged. These findings suggest that even mild maternal thyroid hormone insufficiency could affect early CNS development via dysregulation of BDNF.

Materials and Methods

Animals

Male and female Sprague Dawley rats were purchased from Charles River (Kingston, NY), bred in-house, weaned at 23 d, and housed with one to two others until use. Animals were maintained on a 12-h light, 12-h dark cycle (lights on at 0700 h), with food (Purina 5001; WF Fisher, Somerville, NJ) and water *ad libitum*. Animal care and use were in accordance with procedures outlined in the National Institutes of Health Guidelines. Food and water intake of parents were calculated as the mean intake per day from measurements taken Monday, Wednesday, and Friday throughout the mating and gestation periods.

Thyroid hormone measurements

Trunk blood was centrifuged (2000 RPM, 10 min, 23 C) after 1–2 h at room temperature. Serum was stored at −80 C for later analyses by RIA. Serum concentrations of TSH, free T_3, free T_4, and total T_4 were assayed according to kit instructions (Siemens Healthcare Diagnostics, Deerfield, IL). All samples were run in duplicate. The intraassay coefficients of variation for the TSH, free T_3, free T_4, and total T_4 assays were 6, 7, 5, and 4%, respectively. The corresponding interassay coefficients of variation were 9, 9, 9, and 14.5%, respectively. Based on a 95% specific binding criterion, the sensitivity of the RIA for TSH, free T_3, free T_4, and total T_4 was 0.15 μIU/ml, 0.66 pg/ml, 0.08 ng/ml, and 0.82 μg/ml, respectively. Results below these limits were recorded as the lower limit of detection for statistical analysis.

PTU and BDNF

Virgin males and females were housed together throughout mating, pregnancy, and postpartum. PTU (4 parts per million; Sigma-Aldrich, St. Louis, MO) was made every 1–2 wk, dissolved in distilled H_2O, and stored at room temperature in the dark. Control animals received distilled H_2O. PTU treatment started at the time the dam and stud were first placed together and continued until P (postnatal day) 3 (P0 = day of birth) or 7 d after birth (P7). At approximately the same time of day (1000–1400 h), parents and pups were rapidly (<45 sec) anesthetized by isoflurane inhalation and decapitated. Trunk blood was collected first, and the brain was immediately microdissected afterward (Supplemental Methods and Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Dissections were conducted in the same order (first stud, then dam, then pups). Brain regions were placed in Eppendorf tubes, immersed immediately in liquid N_2, and stored at −80 C. BDNF levels were measured by ELISA (R&D Systems, Minneapolis, MN; see Supplemental Information for details). Assays were conducted blinded to the sex of the pups. Pup sex determination, conducted blinded to treatment, was based on PCR amplification of the male-specific sex determining region of the Y chromosome *Sry* (30) using small samples of subcortical tissue that were frozen immediately after the other brain regions.

Statistical analyses

All values are expressed as mean ± SEM. The α-level for all analyses was set at 0.05. Student's *t* test was two tailed and evaluated using Microsoft Excel (Microsoft Corp., Redmond, WA). A χ^2 analysis used Quantitative Skills software (Hilversum, The Netherlands). ANOVA was conducted using Statview (SAS Institute, Cary, NC). Before ANOVA, Bartlett's test (31) was used to test for homoscedasticity of variance. To minimize the effects of departure from homoscedasticity (in the hormone assay data) data were log transformed before ANOVA.
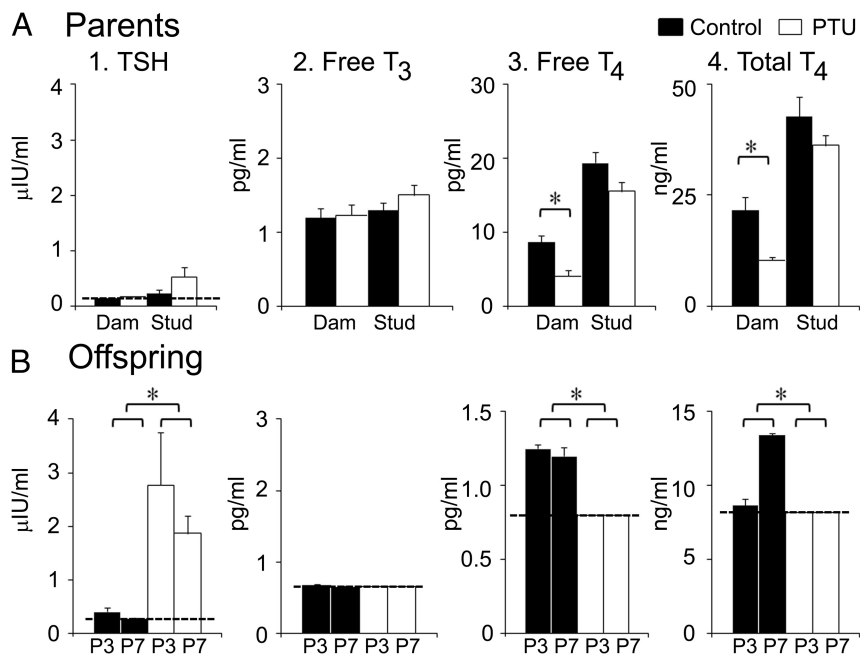
FIG. 1. Thyroid hormone levels in PTU-treated parents and their offspring. A, Parental levels of four hormones were assayed from serum after control (*black columns*) or PTU treatment: 1) TSH, 2) free T_3, 3) free T_4, and 4) total T_4. There were six dams and six studs (from three litters that were euthanized at P3 and another three litters at P7). There were significant effects of PTU treatment on maternal free T_4 (two-way ANOVA, F = 14.423; *df* 1,20; P = 0.0011 followed by a *t*-test of control *vs.* PTU-treated dams, P = 0.004539) and total T_4 (F = 10.144; *df* 1,20; P = 0.0047 followed by a *t*-test, control *vs.* PTU-treated dams, P = 0.009917; Supplemental Table 2A). For TSH and free T_3, there was no effect of PTU (Supplemental Table 2A). B, Thyroid hormone levels of pups born to control and PTU-treated parents. For assays at P3 or P7, sera from six pups were pooled for each litter (P3, n = 6 litters; P7, n = 3). Two-way ANOVA showed significant effects of PTU on TSH (F = 48.817; *df* 1,14; P < 0.0001), free T_4, (F = 9.663; *df* 1,12; P = 0.0090), and total T_4, (F = 61.959; *df* 1,14; P < 0.0001; Supplemental Table 2B). A *dotted line* designates the lower limit of detection of the assays. *Asterisks* indicate significance (P < 0.05).

Results

PTU-treated parents gave birth to more males than females

To evaluate the ratio of females to males in litters of control *vs.* PTU-treated parents, only those litters were used where all pups were sexed (12–13 pups/litter; control: n = 6; PTU: n = 5). Fewer females were born to the PTU-treated parents (control females: 64 of 111 pups, or 57.6%; PTU: 19 of 86, or 22.1%; χ^2, P < 0.000001).

Dams and studs used for the control and PTU groups were not different in either age at the time of mating or the time to mate and deliver, and there were no differences in litter size (Supplemental Table 1). To ensure that differences in food and water intake of control and PTU-treated parents did not influence the results, measurements were made in a subset of the parents (three breeding pairs per group), and there were no significant differences (food intake in grams per day, *t*-test, P = 0.4558; water in milliliters per day, *t*-test, P = 0.0642; Supplemental Table 1).

Weight gain was also not significantly different in these control and PTU-treated parents (Supplemental Table 1).

Serum thyroid hormone levels

Parents

Three-way ANOVA revealed no significant effect of PTU on either TSH or free T_3, but there was a statistically significant reduction in both free and total T_4 (Fig. 1A and Supplemental Table 2A). In the dams, both free and total T_4 were reduced by greater than 50% after PTU treatment (Fig. 1A and Supplemental Table 2A). Significant sex differences were observed in free and total T_4 because of the higher overall values observed in males (Supplemental Table 2A).

Offspring

Hormone levels of offspring were assayed using serum pooled from six pups per litter at P3 (n = 6 litters) or P7 (n = 3 litters). Two-way ANOVA revealed that PTU treatment significantly increased circulating TSH at both P3 and P7 (Fig. 1B and Supplemental Table 2B). Values for free T_3, free T_4, and total T_4 were often below the lower limits of detection of the assays; this was rare in control litters (Fig. 1B). The PTU effect was not statistically significant for free T_3 but highly significant for both free and total T_4 (Fig. 1B and Supplemental Table 2B).

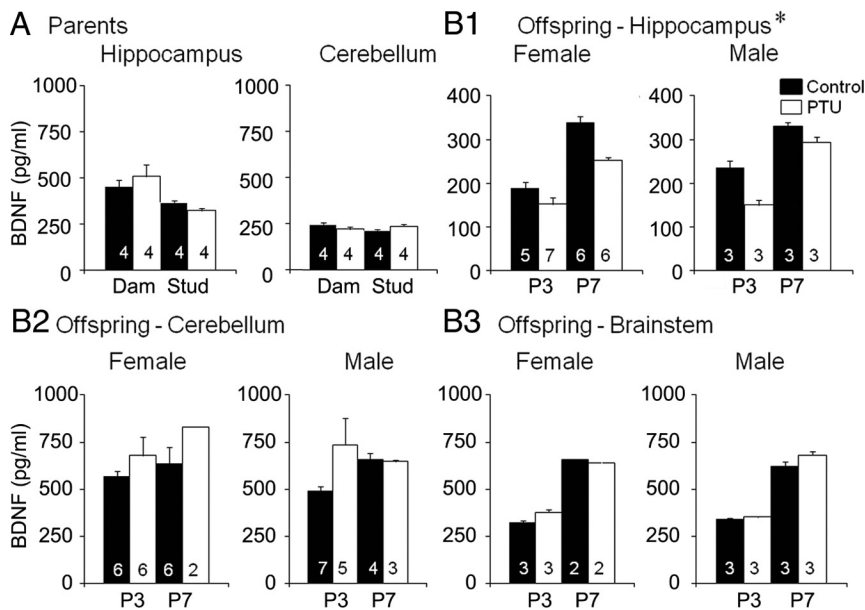### Effects of PTU on BDNF levels in hippocampus

Preliminary one-way ANOVA established that there were no significant litter-dependent effects on BDNF levels in any of the treatment groups. Therefore, data from different litters were combined for subsequent analysis.

Parents

PTU did not significantly affect hippocampal BDNF levels of parents (Fig. 2A and Supplemental Table 3A). A sex difference was noted, reflecting higher hippocampal BDNF levels in the dams (Fig. 2A and Supplemental Table 3A).

Offspring

Hippocampal BDNF levels of offspring were lower in PTU-treated litters compared with controls at both P3 and

FIG. 2. Lower hippocampal BDNF protein levels in offspring born to PTU-treated parents. A, Hippocampal BDNF levels of parents measured by ELISA (control, *black columns*; PTU-treated, *white columns*) showed that there were no effects of PTU on BDNF levels, but fathers had lower BDNF levels than mothers (two-way ANOVA; F = 12.447; *df* 1,12; P = 0.0042 followed by a *t*-test, all dams *vs.* all studs, P = 0.006735; Supplemental Table 3A). For A and B, the *number at the base of the bar* reflects the number of litters. There were no effects of PTU on cerebellar BDNF levels of parents (Supplemental Table 3A). B1, Hippocampal BDNF levels of pups showed that there was an effect of prenatal PTU (three-way ANOVA; F = 27.828; *df* 1,74 P < 0.0001; Supplemental Table 3B). Offspring of parents that were treated with PTU had lower BDNF levels than controls at P3 (*t*-test, P = 0.00001353) and P7 (*t*-test, P = 0.001094). There were no significant effects of sex (Supplemental Table 3B). B2 and B3, There were no detectable effects of PTU on the BDNF levels in the cerebellum or brain stem (Supplemental Table 3B).

P7 (Fig. 2B1 and Supplemental Table 3B). There were no statistically significant sex differences, although the small number of females in PTU-treated litters limits the statistical power of the sex comparison.

Additional analysis of PTU effects

Parents

Other brain regions and serum were sampled in the parents to confirm that their BDNF levels were relatively unaffected by PTU. The pituitary, frontal regions of the brain, and serum showed no significant effect of PTU treatment (Supplemental Table 4).

Offspring

Frontal regions, pituitary, and serum were not possible to assay in offspring, but the cerebellum and brain stem were. Cerebellum BDNF levels tended to be higher at P7 and after PTU treatment, but these trends were not statistically significant (Fig. 2B2 and Supplemental Table 3B). There were no significant sex differences (Fig. 2B2 and Supplemental Table 3B). Mean BDNF levels in brainstem stem increased almost 2-fold between P3 and P7, but

there were no significant effects of PTU (Fig. 2B3 and Table 3B).

Discussion

These results demonstrate that PTU exposure of adult rats, at a dose inducing modest reductions in circulating T_4 concentrations and no significant effect on brain BDNF, significantly altered the thyroid hormones and hippocampal BDNF levels in the offspring at 3 and 7 d after birth.

Although these effects are consistent with our initial hypothesis, the data must be interpreted cautiously. A decrease in hippocampal BDNF does not necessarily imply a decrease in BDNF-dependent function because receptors (*e.g.* trkB, p75) may increase to compensate. Other factors to consider include potential changes in the precursor to BDNF, proBDNF, which plays an important role in development, and members of the sortilin family, which modulate effects of neurotrophins (32). Changes in offspring born to PTU-treated dams may be due not only to direct effects of hypothyroidism but also indirect effects of the PTU treatment in dams, transmitted to the offspring. Although maternal BDNF is unlikely to cross the placenta, other factors do so and could affect fetal development.

Alteration in BDNF levels in early life could contribute to the adverse neurodevelopmental effects that occur after mild prenatal hypothyroidism. This conclusion is consistent with results of previous studies of more severe maternal hypothyroidism (16, 33, 34). For example, Liu *et al.* (16) found that BDNF levels were reduced in the hippocampus of pups born to thyroidectomized mothers implanted with minipumps containing T_4. In the T_4-replaced dams, circulating TSH concentrations remained more than 15-fold higher than those of nonthyroidectomized controls, 1 d after birth, suggesting that the mothers remained severely hypothyroid despite T_4 infusion (16). In the present study, by contrast, there were only modest changes in thyroid status in the PTU-treated dams. In the pups, by contrast, circulating TSH was markedly elevated compared with the offspring of control parents, whereas hippocampal BDNF levels were reduced. The PTU effect may have been more pronounced in the pups because the

rat thyroid gland does not start to develop until shortly before birth, so the fetus is critically dependent on maternal T_4 (1, 35, 36).

A recent study using low-dose prenatal PTU in rats found no detectable effect of PTU on hippocampal BDNF, 14 d after birth (20). Those results are not discordant with the present findings because a transient PTU-dependent reduction in hippocampal BDNF in early postnatal life of rats may be followed by a period of relatively normal BDNF levels. Hormonal effects during early development often remain latent until later in life, when they may re-emerge as changes in hormone sensitivity (37, 38). The results from the present study are consistent with the hypothesis that prenatal PTU exposure may alter BDNF regulation in the brain at a critical stage of early development, the effects becoming fully apparent only later in life. The data of Lasley and Gilbert (20) fit this model: even though in their study BDNF levels in the second and third postnatal weeks were unaffected by fetal hypothyroidism, BDNF protein levels and hippocampal physiology were impaired in adulthood (20, 22).

Similar considerations may apply to the cerebellum. The development of the cerebellum is highly dependent on thyroid hormone and neurotrophins, including BDNF (24, 39, 40). Prenatal hypothyroidism does not produce dramatic changes in cerebellar BDNF expression, however, until well after birth (24, 39, 40). In the present study, cerebellar BDNF was slightly, but not significantly, elevated in the pups. Significant PTU-induced deficits in cerebellar BDNF might not be apparent until much later in development.

A major difference in the experimental design between the present and most previous studies is that the latter have frequently initiated treatments to induce hypothyroidism only toward the end of the first week of pregnancy or later (2, 20). Our animals were treated during mating and conception and for the entire duration of pregnancy. This is likely to have been the reason for the unexpected differences we observed in sex ratios of offspring because dams and studs were exposed to PTU when the sex of the offspring was being determined. Interestingly, other recent studies also suggest that thyroid hormone may influence sex ratios. In amphibia, thyroid hormone affects gonadal sexual differentiation (41), whereas in bovine embryos developing *in vitro*, the addition of T_3 and T_4 increases the number of females reaching the blastocyst stage of development (F.A. Ashkar and W.A. King, personal communication).

In summary, these results support the view that low doses of PTU throughout pregnancy represent a valuable model to evaluate the effects of fetal hypothyroidism and suggest that measurements of neurotrophin levels as well as neurotrophin-mediated responses could be useful in assessing the potential effects of prenatal exposure to drugs and chemicals that interfere with thyroid function. Such exposure may not be reflected in detectable changes in maternal indices of thyroid function and, moreover, may be apparent only transiently in the pups, even under conditions in which there may be lasting developmental effects on the brain (20).

Acknowledgments

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